Terminaliabellerica (BAHEDA) INHIBITS PROTECTIVE AUTOPHAGY AND INDUCES APOPTOSIS IN ORAL CANCER CELL LINES

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CERTIFICATE

This is to certify that the thesis entitled **"*Terminalia bellerica* (baheda) inhibits protective autophagy and induces apoptosis in oral cancer cell lines"** which is being submitted by Ms. Priyadarshini Padhi, Roll No- 412LS2047, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

SKBhutia
Sujit K Bhutia

Dedicated to my beloved parents

DECLARATION

I do hereby declare that the Project Work entitled **"*Terminaliabellerica*(baheda) inhibits protective autophagy and induces apoptosis in oral cancer cell lines"**, submitted to the Department of Life Science,National Institute of Technology, Rourkela is a faithful record of bonafide and originalresearch work carried out by me under the guidance and supervision of Dr.Sujit KumarBhutia, Asst. Professor, Department of Life Science, National Institute of Technology,Rourkela, Odisha.

Date: 11/05/2014

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LIST OF ABBREVIATIONS

PBS	Phosphate Buffer Saline
et al	And others
Conc	Concentration
Hrs	Hours
pH	Hydrogen concentration
ROS	Reactive Oxygen Species
DAPI	Diamidinophenylindole
TBE	*Terminalia bellerica* extract (water extract)
AIF	Apoptosis Inducing Factor
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
DMEM	Dulbecco's modified Eagle Medium
MEM	Minimum essential medium
%	Percentage
mg	Milligram
µg	Micro gram
nm	Nano meter
µM	Micro molar
Ml	Micro liter
Atg	Autophagy gene
ER	Endoplasmic reticulum
ROS	reactive oxygen species
mTOR	Mammalian target of rapamycin
LC3	Light Chain 3
DISC	Death inducing signal complex
Mitophagy	Mitochondrial autophagy

1. ABSTRACT

Cancer is a dreadful disease being one of the leading causes of death throughout the world. It has been estimated that about 8.2 million deaths in 2012 is caused by cancer only. In India oral cancer is the most common cancer prevailing among the individuals irrespective of their socio-economic status. There are many causes of oral cancer with many complex mechanisms of their originality and functionality. Among many mechanisms of cancer proliferation, metastasis and their drug resistance capacity, deregulations in the cell death mechanism is one. There are three types of cell death machinery and these are apoptosis, autophagy and necrosis. The complex interplay of autophagy or apoptosis with tumor cells has been reported by many scientists which are rather very confusing and also contradictory form one type of cancer to another. In this study there the aim is to find out potential anticancerous property in a fruit *Terminalia bellerica* and its cytotoxic effects on oral cancer cells. Two cell lines : FADU and Hep 2 are taken for the study . Various tests have been performrd in this study to detect the apoptotic, autophagic and DNA damaging properties of *T. bellerica* and the results shows that it has potential anticancerous properties which can inhibit protevtive autophagy and promotes apoptosis in Hep 2.

2. INTRODUCTION

Tumor cells are characterized by several hall marks such as limitless growth, not sensitive to antigrowth signal,extended angiogenesis,limitless replicative potential,skipping cell death, metastasis as well as get rid of immunological surveillance[1].According to the report of National Institute of Public Health (Japan) in February 2011, 86% of the world's oral cancer victims reside in India which signifies India is hub of oral cancer[2]. After failuring from various chemotherapeutic treatment and drug resistance herbal extract came into focus taking its past use as tradicinal medicine. Therefore *Terminalia bellerica* water extract was taken as an potential anticancer weapon specifically in human larynx carcinoma cell line (Hep 2). This is the first report *Terminalia bellerica* water extract inhibits protective autophagy and augments apoptosis in (Hep 2).

Due to the increasing failure of chemotherapeutics and antibiotic resitance exhibited by pathogenic microbial infectious agents temted to investigate several medicinal due to their active components which could combat cancer. *Terminalia bellerica* Roxb., belongs to family 'Combretaceae' which is a large deciduous tree commonly found in the plains and lower hills of Southeast Asia. Phytochemical studies explained that T. bellerica contains several active chemical components including, ellagic acid, tannins, gallic acid, 2, 3, 7, 8-tetraoxy-chromeno[5,4,3-cde]chromene-5,10- dione and Epigallocatechin gallate [3]. Several antiproliferative reports are also available for *Terminalia bellerica* as it shows cytotoxicity in various cell lines like Shionogi 115, PC-3,DU-145 , MCF-7. *Terminalia bellerica*is also main component of triphala along with *Emblica officinalis* and *Terminalia chebula*. *Terminalia bellerica* decreases protective autophagy in Hep 2 cell line on the contrary apoptosis increases.

Therefore an attempt has been made to draw a connecting link between autophagy and apoptosis which were induced by *Terminalia bellerica* water extract in human larynx carcinoma cell line (Hep 2).

3. REVIEW OF LITERATURE

3.1. Autophagy

Autophagy (type II cell death mechanism) is a highly regulated self-degradative pathway in all eukaryotic cells, in which lysosomal degradation of damaged and superfluous cytoplasmic components occur, and so, basic biomolecules are produced which are recycled back into the cytosol for generation of new macromolecules. These recycled products are also utilized as alternative energy sources for the cells undergoing metabolic stress like infection, nutritional starvation, hypoxia, and intracellular stress like, piled up damaged proteins and organelles and also during high bioenergetic demands etc, to maintain cellular homeostasis. In tumour cells with defective apoptosis, autophagy helps in prolonged survival of the cells. In some cases, defective autophagy also induces increased tumorigenesis. Also in some recent studies it has been seen that autophagy helps in protecting tumour cells from inflammation due to necrosis and alleviating genome damage in cancer cells during metabolic stress. Thus, autophagy is generally considered as cell survival mechanism in physiological and pathological conditions such as neurodegeneration, myocardiopathy, cancer etc, although it has been proved to regulate apoptotic as well as non-apoptotic cell death.

The cellular catabolic degradation pathway in autophagy is highly conserved in all eukaryotic cells which is executed by autophagy related effectors, many of these known as ATG proteins [4, 5]. It is performed in three main steps: nucleation, elongation, and degradation. Autophagy begins with formation of an isolation membrane known as phagophore. First a

cellular stress inactivates a stress sensor, mTOR (i.e. mammalian target of rapamycin), resulting in hypophosphorylation of Atg13. Then with the help of Atg17, this Atg13 binds to Atg1 (mammalian homolog of Ulk1), which subsequently recruits Atg9. Atg9 extracts lipids from various sources to form phagophore [6]. The exact origin of phagophore is controversial; however, it is likely derived from the lipid bilayer of ER and/or trans-Golgi and endosomes [7, 8]. This process is known as vesicle nucleation which is executed by a protein complex. The protein complex core comprises of (i) the class III phosphatidylinositol-3-kinase (PI3Kc3 or VPS34) which catalyzes phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P), (ii) the PI3Kc3 regulatory subunit (p150 or Vps15), a myristylated serine/threonine kinase which phosphorylates PI3Kc3 and recruits it to the membrane, and (iii) the BCL-2 interacting protein, Beclin 1 (the mammalian homolog of yeast ATG6), which likely acts as a protein interaction hub [9]. Ambra1, another part of the core complex, acts as a positive regulator of autophagy that interacts with Beclin 1 [10]. Further the core complex associates with various other proteins that have distinct role in membrane trafficking processes. These proteins are ATG14, UVRAG (UV radiation resistance-associated gene), Vmp1 (vacuole membrane protein 1), Bif-1 (endophilin B1), and Beclin -1 associated RUN domain containing protein (Rubicon) [11, 12].

In the next step, membranes comprising the nascent phagophore get enlarged and then fuse at their edges to form multi-layered vesicles known as autophagosomes. Formation of autophagosome comprises of two ubiquitin like systems [13]. In one system, Atg 5- Atg 12 conjugate, where Atg 12 has an ubiquitin like fold, is formed by two enzymes, Atg 7 (an E1 like ubiquitin activating enzyme), and Atg 10 (an E2 like ubiquitin carrier protein or enzyme). Then Atg5-Atg12 complex non-covalently conjugate with Atg16L dimers to form a

multimericAtg5-Atg12-Atg16 complex. The Atg5-Atg12-Atg16L complex seems to associate with the extending phagophore to induce curvature into it [14]. In the other system, another ubiquitin-like protein, ATG8/LC3 is involved, which is a microtubule-associated protein light chain, encoded by the mammalian homologue of Atg8. LC3 is conjugated to phosphatidylethanolamine, by the sequential action of ATG4 (a protease), ATG7 (an E1-likeenzyme), and, ATG3 (an E2-like enzyme). Recruitment and integration of LC3B-II into theextending phagophore is dependent on Atg5–Atg12 conjugate and LC3B-II is found on both the internal and external surfaces of the autophagosome, where it plays a role in hemifusion of membranes and also in selecting cargo for degradation [14].

After formation of autophagosome, it fuses with lysosome to form autophagolysosome. This process involves specific ATG8 paralogs; multiple WD repeat (i.e. tryptophan-aspartic acid repeats) domain containing, phosphoinositide-interacting ATG18 paralogs; ATG2A, which binds to ATG18; the transmembrane protein, ATG9; SNX18 which causes autophagosometubulation; as well as small G-proteins that signal this process. The contents of the autophagosome are degraded by lysosomal enzymes inside the autophagolysosome, and after this, the degradation products are recycled by the cell [9].

Role of autophagy at different stages of cancer.

Autophagy generally has double-edge role in cancer development [15]. Depending on the cell type and stage of tumor development, autophagy may be protective or lethal. In protective autophagy, tumor cells undergo enhanced proliferation and metastasis by escaping the environmental stresses like anoikis, the first step of metastasis of cancer cells [16] and also in other stress conditions like nutrient poor and hypoxic conditions. Protective autophagy maintains

TRAIL-resistant phenomena and , altered energy metabolism of tumor cells during tumor progression. Tumor cells maintain protective autophagy by activating HIF-α (hypoxia-inducible factors) and AMPK (50-AMP-activated protein kinase) in apoptosis deficient (due to mutation in the tumor suppressor genes, and/ or oncogenes) and long-term metabolic stress conditions, in a fully metastatized cancer [17]. Paradoxically, autophagy-related cell death has been regarded as a primary mechanism for tumor suppression during initial stages of cancer development. Moreover, autophagy also restricts metastasis of tumor cells at a premature step by restricting necrosis and inflammation of developing tumor, thus limiting their invasion and dissemination from primary site or the site of their origin. Moreover, lethal (toxic)autophagy brings about the release of immunomodulatory factors such as HMGB-1 from dead tumor cells, which triggers immune response and restricts metastasis by inhibiting pro-tumorigenic responses [17].

3.2. Apoptosis

Apoptosis, or type 1 form of programmed cell death, is another cellular process triggered by various strictly conserved cellular signals in healthy individuals. Apoptosis is responsible for many biological processes such as normal cell turnover, hormone-dependent atrophy, proper development and functioning of the immune system, and embryonic and neural development. So in general, it performs as a homeostasis mechanism as well as a defence mechanism. Inappropriate apoptosis (either too little or too much) is a contributing factor in many human pathophysiological conditions including neurodegenerative diseases, vascular and cardiac diseases, autoimmune disorders and many types of cancer. Apoptosis is characterised by several cellular changes, like altered nuclear morphology including chromatin condensation and DNA fragmentation, minor changes in cytoplasmic organelles, cell shrinkage, plasma membrane blebbing, and apoptotic body formation. Apoptosis is triggered by many signals, either in

intrinsic/intracellular/mitochondria mediated pathway, or extrinsic/extracellular/receptor mediated pathway. Caspases are the executioners of apoptosis which cleave about 400 proteins and bring about the morphological changes and death in apoptotic cells [18]. These apoptotic caspases are of three types: "initiator caspases" (caspase-2, −8, −9 and −10) that initiate an apoptotic cascade and "effector caspases" (caspase-3, −6 and −7) that disassemble the cell, and "inflammatory caspases" (caspase-1, -4, -5) [14, 16]. These are controlled by specific cellular inhibitors known as inhibitor of apoptotic proteins (IAP), which bind to caspases and inhibit them. To overcome the inhibitory effect of IAPs, Smac/DIABLO and HtrA2/Omi are released from the mitochondria and bind to or cleave IAPs, respectively [19].

Intrinsic pathway:

The intrinsic pathway is triggered by various intracellular apoptotic signals like hypoxia, oxidative stress, DNA damage, and growth factor deprivation leading to permeabilization of outer mitochondrial membrane [20]. This mechanism is regulated by members of Bcl-2 family of proteins. In general, the members of the Bcl-2 family have been grouped into 3 classes: the first class includes anti-apoptotic BH1234 proteins (e.g. Bcl-2, Bcl-xL, and Mcl-1) which inhibit apoptosis; the second class includes pro-apoptotic BH123 proteins which promotes apoptosis (e.g. Bax and Bak); and the third divergent class of BH3-only proteins which sense different kinds of cell stress/damage, (e.g. Bad and Bid) [21]. The initiation of intracellular signals which stimulates the intrinsic pathway may either be in positive or negative fashion. When the cell experiences negative signals like deficiency in growth factors, hormones and cytokines, the cell fails to suppress death programs and undergoes apoptosis, whereas stimuli like radiation, hypoxia, hyperthermia, toxins, free radicals and viral infections act in positive fashion to induce apoptosis. This can be explained at molecular level as: in one way, apoptotic signals (in negative

fashion) can activate the BH3-only proteins, thereby the antiapoptotic Bcl-2 proteins are inactivated. This relieves inhibition of the proapoptotic Bcl-2 proteins and induces apoptosis. In contrast, the opposing model (i.e. apoptosis in positive fashion) postulates direct activation of pro-apoptotic proteins, Bax and Bak by several BH3-only proteins. The proapoptotic members of the Bcl-2 family of proteins enhance the permeability of the mitochondrial outer membrane which is known as MOMP (mitochondrial outer membrane permeabilization) [22]. An increase in MOMP leads to release of mitochondrial intermebrane protein, including apoptogenic molecules such as cytochrome c, to the cytoplasm. In the presence of ATP, cytochrome c binds to Apaf-1, i.e. apoptotic protease activating factor-1, and triggers oligomerization. This complex, known as an apoptosome, a seven-spoked wheel-shaped complex, recruits and cleaves procaspase 9 which gets converted into the active enzyme, caspase 9, which, in turn, activates caspase 3, the enzyme that is directly responsible for cell death.

Extrinsic pathway:

The extrinsic apoptotic pathway is activated by trans-membrane receptor mediated interaction in which death receptors (DR) present on the cell surface bind to specific apoptotic signal transmitting ligands. Such ligands involve soluble molecules of the TNF (tumor necrosis factor) family, which are secreted as homotrimers and bind to members of the TNF-R (TNF-receptor) family, including TNFR-1, and TRAIL receptors (TNF-α-related apoptosis-inducing ligand) DR-4and DR-5, Fas/CD95. When the ligand binds to receptor, the receptor undergoes trimerization and subsequent activation [23, 24]. TNF-receptors (TNF-R) possess a death domain (DD), which recruits other DD-containing proteins, such as TNF-R type 1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD). These proteins bind to initiator caspases-8 and -10, thus resulting in the homodimerization and then subsequent

activation of death-inducing signalling complex (DISC) [25, 26]. After the activation of caspases-8 and -10, the caspases-3, -6, and -7 (effector caspases) cleaved, eventually bringing cellular degradation and so, induce apoptosis [27].

3.3. Relation between autophagy and apoptosis in cancer development

Two opposite situations may arise due to the intricate interaction between the autophagic and apoptotic mechanism. These are, inhibition of autophagy may result apoptosis, and suppression of apoptosis may cause autophagy [28, 29, 30]. Alteration in autophagy may have differential effects on cancer cells. When autophagy is efficient, it helps in survival of cancer cells. When autophagy is inefficient or deregulated, it can induce either apoptotic or non-apoptotic (necrosis or Programmed cell death type II) to the cancer cell. So, it has been proposed by many scientists that, by manipulating autophagy, thus regulating or deregulating the relation between autophagy and apoptosis in tumour cells, we can fight cancer.

3.4. *Terminalia bellerica :*

Terminalia bellerica (common name is Baheda in Hindi ,in India) is widely distributed in Sri Lanka, India and Nepal, Burma (Myanmar), Indo-China and Thailand, Penninsular Malaysia, Sumatra, Java, Borneo (Sabah), the Lesser Sunder Island, central Sulawesi and the Moluccas [31]. So, basically the plant is found in south-east Asian countries and is popularly used as traditional medicine. Its tremendous medicinal properties are used in Indian ayurvedic medicine. Its fruit is commonly used as a part in the medicine '*Triphalachurna*' in combination with *Terminalia chebula* (Harida), and *Emblica officinalis* (Amla).

Phytochemicals

Terminalia bellerica contains the following phytochemicals: Glucoside (bellericanin) [5, 32], coloring matter, resins and a greenish yellow oil, Gallo-tannic acid [40], Ellargic acid, gallic

acid, lignans(termilignan and thannilignan), 7-hydroxy 3'4' (methylene dioxy) flavone and anolignan B [32]. Tannins, ellargic acid, ethyl gallate, galloyl glucose and chebulaginic acid, phenyllemblin, β-sitosterol, mannitol, glucose, fructose and rhamnose [33, 34, 5, 35].

Classification of *Terminalia bellerica*(Gaertn.)Roxb.(according to the GBIF Taxonomy Hierarchy) [20]

Kingdom:	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia*
Species	*bellirica*

Synonym: Myrobalanusbellirica

Figure 1: *Terminalia bellerica*

Current studies on the anti-cancer properties of *T. Bellerica* proves it to be an useful asset for preparing a potential anti-cancer drug. In one study, in vitro experiment was carried out to know about the anticancer activity of 70% methanolic extract of Terminalia bellerica (TBME)

against human breast (MCF-7) carcinoma and human lung (A549) carcinoma and its possible mechanism [21]. The extract showed significant cytotoxicity to both A549 and MCF-7 cells, whereas, in non-malignant WI-38 cells no cytotoxicity was found. Flow cytometric analysis of A549 and MCF-7 was carried out by taking 100 µg/ml of TBME as the effective concentration inducing apoptosis in the cancer cell lines. This concentration of TBME proved to cause DNA fragmentation pattern of apoptosis. To know the mechanism of apoptosis induction western blotting was performed in which the ratio of Bax/Bcl-2 in both A549 and MCF-7 was found increased, which in turn activates the caspase cascade and the cleavage of PARP. These results confirmed the anticancer effects TBME in both lung and breast cancer cell lines by modifing the Bcl-2 family proteins [21].

Pinmai K et al. (2008) studied the growth inhibitory activities and synergistic effects of *P. emblica*extract/ doxorubicin or cisplatin and *T. Bellerica* extract/ doxorubicin or cisplatin extracts having certain selective degrees against A549 and HepG2 cells (two cancer cell lines). The specific formulation of appropriate dose level of the combinations with significant antagonistic effects was different for different cell lines [39].

In another study, the antimutagenic effect of two polyphenolic fractions (TB-3, TB-4) isolated from *T. bellerica* in two strains of *S. typhimurium* (i.e. TA98 and TA100) against NPD (4-nitro-o-phenylenediamine), 2AF (2-aminofluorene) and 4NQNO (4-nitroquinoline-N-oxide) has been distinguished [40]. Both the compounds were found to be considerably effective against S9-dependent 2AF; rather less striking effect against NPD and almost no effect against 4NQNO in TA100 strain. The 13C-NMR spectral analysis, of TB-3 fraction identified it to be a mixture of three tannins, and TB-4 was found to have non-tannin fraction. Among many possible

mechanisms of the effectiveness of these polyphenols, the ruled out mechanism was the interaction between the polyphenols with S9 proteins which has some inhibitory effects [40].

It has also been observed that leaf extracts of *Terminalia bellerica* have considerable anticancer compounds with IC50 of 5.65µg/ml [41]. *T. bellerica* has a variety of phytochemicals having various therapeutic applications which have been already reported. Phytochemical studies have revealed that *Terminalia bellerica* contains a variety of bioactive ingredients with reported therapeutic activities. Dinesh M.G *et al.* has reported the dose dependent growth inhibitory activity of Methanolic Terminalia Leaf Extracts (MTLE) against cell line Hep G2 cell line. MTLE supplementation has down regulated expression of Bcl-2 a potent suppressor of apoptosis and induced expression of pro apoptotic proteins Bax, caspase-9 and caspase-3 in a dose dependent manner. The researchers also demonstrated that down regulation of the Akt/mTORsignaling pathway is responsible for expression of Bax.. The percentage of arrest at G2/M phase in Hep G2 cell line increased in comparision to control cell line which. Overall, these findings proves that leaves of Terminalia can act as an anti-cancer drug [41].

4. OBJECTIVES

1. Preparationof *Terminalia bellerica* water extract (TBE).
2. Detecting the antiproliferative activity of *Terminalia bellerica* water extract
3. To check whether TBE induces apoptosis in Hep 2 or not
4. To check whether TBE induces aautophagy in Hep 2 or not
5. To check whether TBE induces DNA damage in Hep 2 or not

5. MATERIALS AND METHODS

5.1. Reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliu (MTT), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO), propidium iodide (PI), trypsin, agarose, Acridine orange (AO), dihydrorhodamine, Low melting Agarose from Sigma Aldrich, India. Fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), antibiotic-antimycotic (100 X), Lipofectamine 2000® were procured from Invitrogen, India. Caspase 3/7 glo kit, from Promega, India. Other chemicals which were used are of analytical grade and high quality.

5.2. *Terminalia bellerica* water extract

Dry fruits of *T. bellerica* were purchased from local market. After that seeds were washed with distilled water. Seeds were soaked in Phosphate buffer saline (PBS) (pH-7.4) over night in 4^0C. After that soaked seeds were grinded and centrifuged at 7500 rpm 4^0C. Pellets were discarded and supernatant were filtered by Whatman® fliter followed by 0.22 µ syringe filter. The filtrate samples were collected in a round bottom flask and stored in -80^0C before lyophlization. After lyophilisation the powered extract were stored in -20^0C. Samples were dissolved for treatment in cell lines.

5.3. Cell lines

Human squamous carcinoma cell line (FADU), and human larynx carcinoma cell line (Hep 2) were cultured in Minimum Essential Medium supplemented with antibiotic antimycotic(1X) and 10 % fetal bovine serum.

5.4. Cell survibility Assay MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide):

Principle :

MTT assay is a colorimetric assay which measures the reduction of yellow coloured 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple coloured formazan product by the mitochondrial enzyme succinate dehydrogenase. So, when the MTT enters the cells and passes into the mitochondria it gets reduced to an insoluble, dark purple coloured formazan product only in a live cell. When solubilised by DMSO (an organic solvent), it imparts purple coloured with the solubilisation of formazan. Then the absorbance of the solution is measured spectrophotometrically at 500- 600 nm.

Protocol :

The cells (Hep 2 and FaDu) were subcultured and when cells reached 80-90% confluency, they were trypsinized. The cells were seeded in 96 well plates. After 24 hours, the cells were treated with different concentrations of TBE (*T. bellerica* extract) and incubated for 72 hours. Then MTT was added to each well. After 4 hours, the formazan was dissolved with and the absobance was measured at 562nm using micro-plate reader spectrophotometer (Perkin-Elmer) [39]

5.5. DAPI (4' ,6-Diamidino-2-phenylindole dihydrochloride) staining of Hep 2 cells

Principle:

DAPI is a nucleic acid stain which when binds to the AT rich region in the minor groove of a dsDNA possibly due to removal of water molecules from both DAPI and dsDNA. With this

reaction DAPI fluoresce blue colour. It basically indicates the chromatin condensation in the nucleus due to apoptosis.

Protocol:

The preseeded cells were treated with different doses of TBE for 24 hr .After that both treated and control cells are fixed with 3.7% paraformaldehyde for 20 mints followed by permeabilization with 0.1% triton X . Cells were stained with 1µg/ml DAPI for 7 mints. After three times washing with PBS, images were taken in the fluorescence microscope.[40]

5.6. DNA laddering assay:

Principle:

Chromosomal DNA fragmentation is one of the major characteristic of apoptosis. This phenomenon is known as DNA laddering. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl termini in the DNA. the 3'-hydroxyl ends of the fragmented DNA can be labelled with conjugated fluorescein-deoxyuridine triphosphate nucleotides (FITC-dUTP) to detect apoptosis in a cell.

Protocol:

DNA fragmentation assay by non-enzymatic method was done to detect the presence of apoptotic death of cells due to the genotoxic effect of TBE. First, the Hep 2 cells were seeded in 60 mm petri-plate. Then one petriplate was taken for control, without any TBE treatment and other three petri-plates were taken for treatment with different doses of TBE. After 24 h incubation, cells were dislodged and pelleted down. Then followed by addition of100 µl DMSO and equal amount of Tris-EDTA buffer (pH) with 2% SDS was added to each of the cell samples and mixed well. Then, the mixture was vortexed for through mixing and then

centrifuged at 12,000 rpm at 4^0C. after this, 40 µl from the resulting supernatant was collected and then it was loaded on 1.5% agarose gel on glass slides. Ethidium bromide was used to stain the DNA and visualized in BioRad gel documentationsystem using Quantity One software.

5.7. Caspase activity assay:

Principle:

Caspase -3 and -7 are the executioner of apoptosis. In this assay, the reagent which is used contains aproluminiscentcaspase 3/7 substrate having DEVD (a tetrapeptide sequence). DEVD, is optimized for luciferase activity, caspase activity and cell lysis.Thecaspase 3/7 reagent lyses a cell and if the cell is in apoptotic stage, then the caspase 3/7 present in the cell cleaves DEVD and produces free aminoluciferin. Aminoluciferin is now acted by luciferase and gives a glow type luminescent signal. So, luminescence is directly proportional to the caspase activity.

Protocol:

Hep 2 cells were seeded in 60 mm plates and TBE were added in various doses , After 24 hr cells were scraped and lyzed and centrifuged in 4^0C at 12000 rpm. After preparing sample caspase activities were measured following the manufacturer's protocol (Promega Corp., Madison, WI) [41].

5.8. Acridine orange staining of Hep 2 cells for detection of autophagy:

Principle:

AcridineOrange is an acidotropic dye used for detection of autophagy. It fluoresces green at neutral pH, but when enters acidic vesicles, it gets protonated, accumulated inside the vesicle, and gives bright red colour fluorescence. In case of late autophagic vacuoles, it enters the autophagolysosome, and becomes protonated, trapped inside it and gives bright red flurescence (due to the acidic pH of the autophagolysosome).

Protocol:

Hep 2 cells were cultured and various doses of TBE were treated for 24 hr. After that cells were were treated with 0.5 µg/ml of acridine orange for 15 min followed by three times washing with 1X PBS. Images were captured in the flurescence microscope. Red intensity were measured in Image J software [42].

5.9. Alkaline COMET Assay:

Principle :

Alkaline Comet assay is a very sensitive assay to detect and quantify even small amount of dsDNA damage in individual cell. It is also known as single cell gel electrophoresis (SCGE). Individual cells are fixed in a thin agarose on a glass slide. From the cell, all the cytoplasmic proteins are removed by cell lysing .In alkaline conditions, the DNA unwinds and then this unwound DNA is allowed to run in a gel by electrophoretic method. Now, the damaged DNA start to migrate from the nucleus from negative to positive pole in the gel cast and form the tail part of the comet whereas the intact DNA form the immobilizing head part of the comet. Then the DNA is stained with propidium iodide (a DNA specific stain) and observed for the amount of red fluroscent in the head, tail and tail length region. The more damage in the DNA the more will be the accumulation of DNA at tail region.

Protocol:

Hep 2 cells were treated with various doses of TBE. TBE treated cells along with the control cells were embedded on the slides with gel and the slides were taken for electrophoresis. After the TBE treated cells and the control cells were lysed with alkaline A1 lysis solution followed by A2 rinse and electrophoresis solution. After electrophoresis was done pictures were captured in flurescence microscope. The tail length increase with increasing dose of TBE.

5.10. Determination of reactive oxygen species (ROS) by dihydrorhodamine:

Principle :

Dihydrorhodamine 123 is an chemical which can indicate the presence of reactive oxygen species (ROS) in a cell. It does not fluoresce when it is uncharged. But when it gets protonated by ROS, it fluoresces green light. It can diffuse across the membrane passively and if oxidised to cationic rhodamine 123 by ROS, then it gets confined to mitochondria and emits green fluorescence.

Protocol :

Hep 2 cells were treated with various doses of TBE and after 24 hr cells were treated with2.5 µg/ml Dihydrorhodamine123 (Dhr123). Then the cells were incubated for 30 mint. After that cells were washed three times with 1X PBS and pictures were captured in flurescence microscope. The green intensity increases with increasing reactive oxygen species [43].

6. RESULT AND DISCUSSION

6.1. Cell viability assay (MTT Assay):

MTT assay was performed to check the viability of two different oral cancer cell lines: FaDu and Hep 2 at different concentrations of TBE.

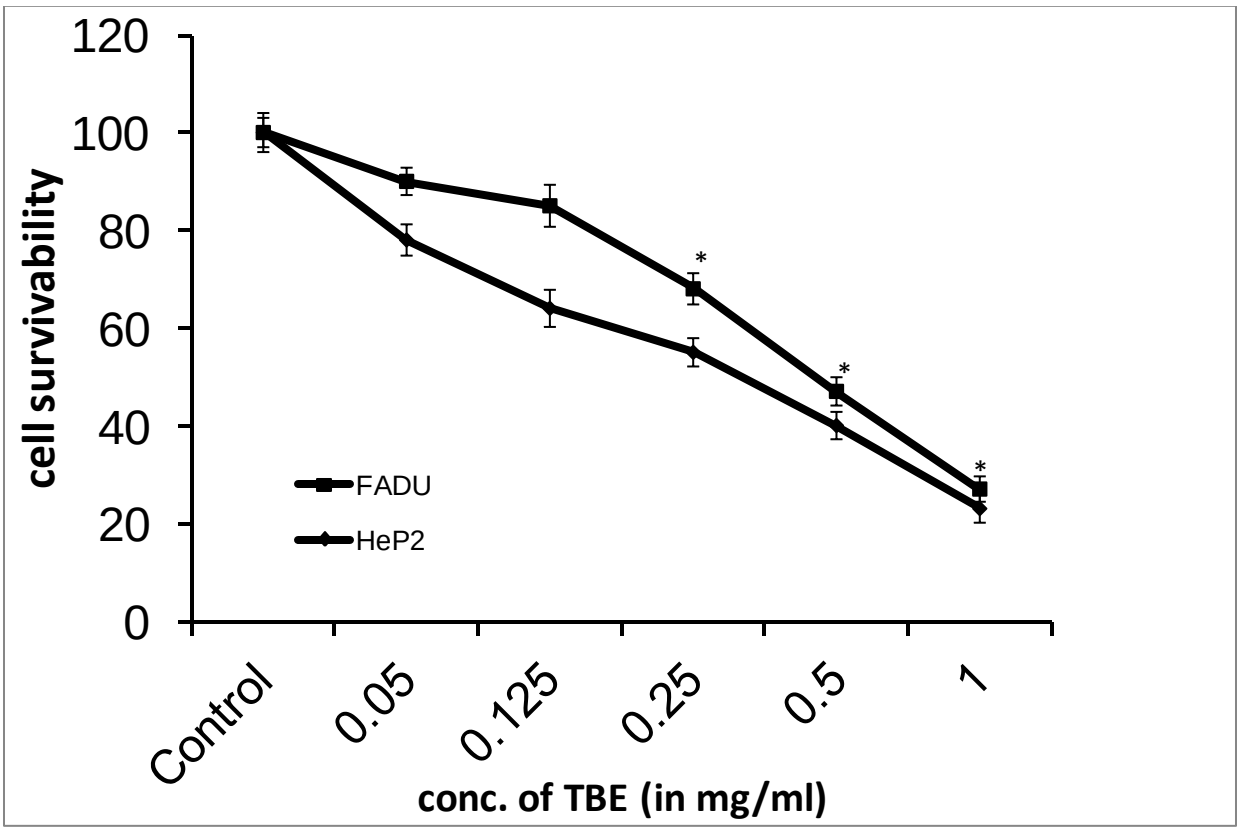


Fig- 2 : Graph showing MTT assay of FaDu and Hep 2 cell lines

Inference:

It was observed that, the cell survivability is decreasing with increase in the concentration of TBE in both the cell lines. The significant concentrations of TBE (the star marked conc. on the graph)at which cell viability is significantly decreasing are 1mg/ml, 0.5mg/ml, and 0.25mg/ml. The graph of MTT assay shows that the TBE has cytotoxic effects on both FaDu, and Hep 2, but

Hep 2 is comparatively more susceptible to decreased cell viability than FaDu at same concentrations.

6.2. DAPI staining

DAPI staining of control and TBE treated of Hep 2cellss was performed to detect nuclear membrane bebbling which is a distinguished characteristics of apoptotic cell death.

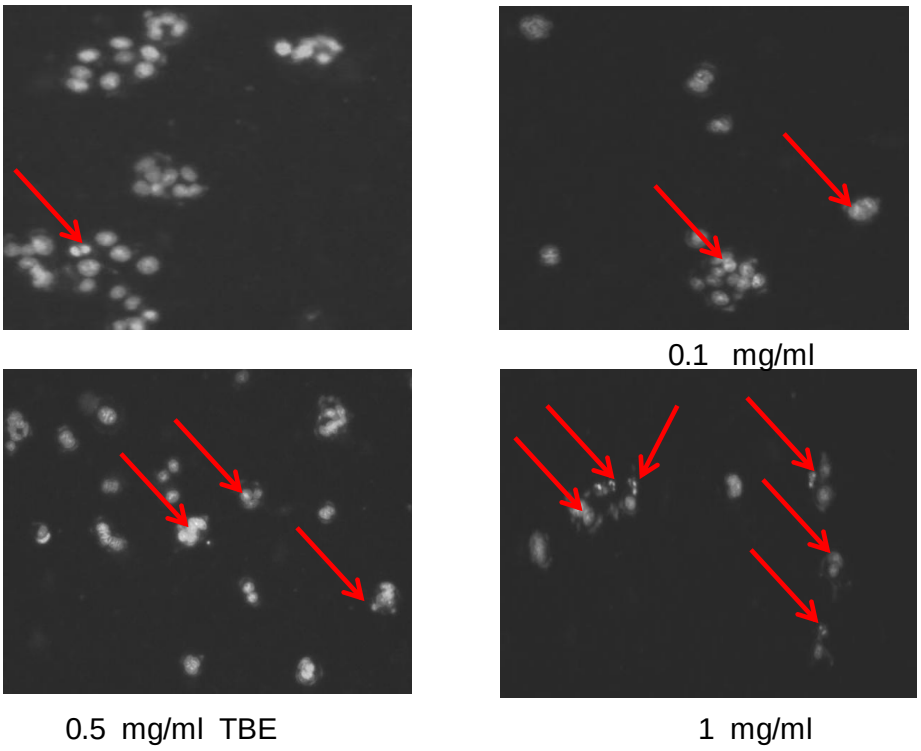


Fig-3: DAPI staining of Hep 2 cells treated without and with different concentrations of TBE.

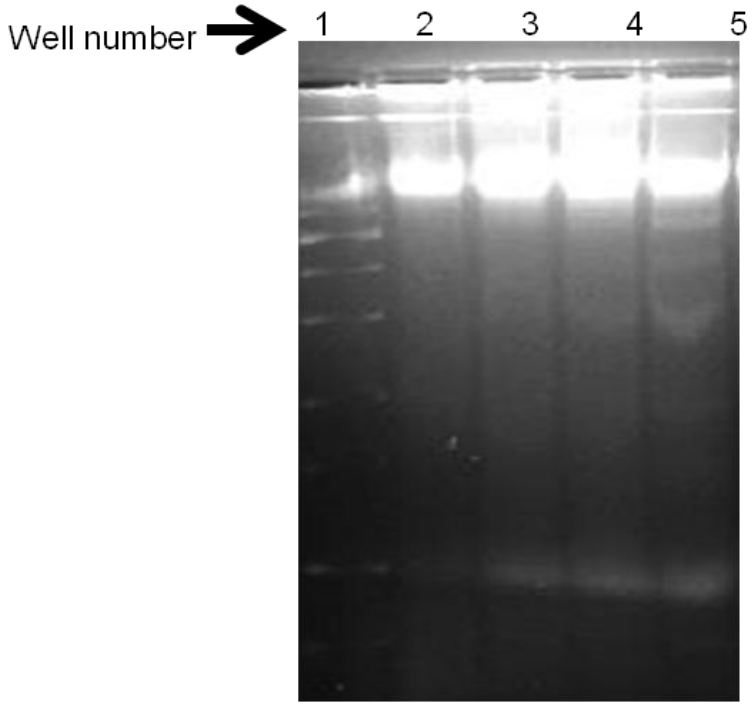
Inference :

From the above figure, significant increase in the nuclear membrane bebbling can be demarked in a dose dependent manner. So, it confirms about the apoptotic cell death of Hep 2 cells is

increasing with increased nuclear membrane blebbing and DNA damage with increase in dose of with increasing dose of TBE.

6.3. DNA laddering test:

In DNA laddering test, the aim was to detect the fragmentation of DNA, a marked characteristics of apoptosis due to effects of TBE.



Well no.	TBE conc (in mg/ml)
1	Marker DNA
2	control
3	0.1
4	0.5
5	1

Fig-4: The gel elctrophoresis shows bands of fragmented DNA of control and TBE treated Hep 2 cells in different lanes

Inference: From gel electrophoretic analysis, it was observed that, the number of DNA bands are increasing with increase in the concentration of the TBE in Hep 2 cells.

6.4. Caspase 3/7 glo assay:

Caspase 3/7 glo assay was performed to detect the caspase activity of TBE treated Hep 2 cells in comparision to control cells.

Table-2: Caspase 3/7 activity of Hep 2 cells when treated with different concentrations of TBE

Conc. of TBE (in mg/ml)	Caspase 3/7 activity (in terms of luminescence of the TBE treated cells)
Control	2096
0.1	3291
0.5	4429
1	7924

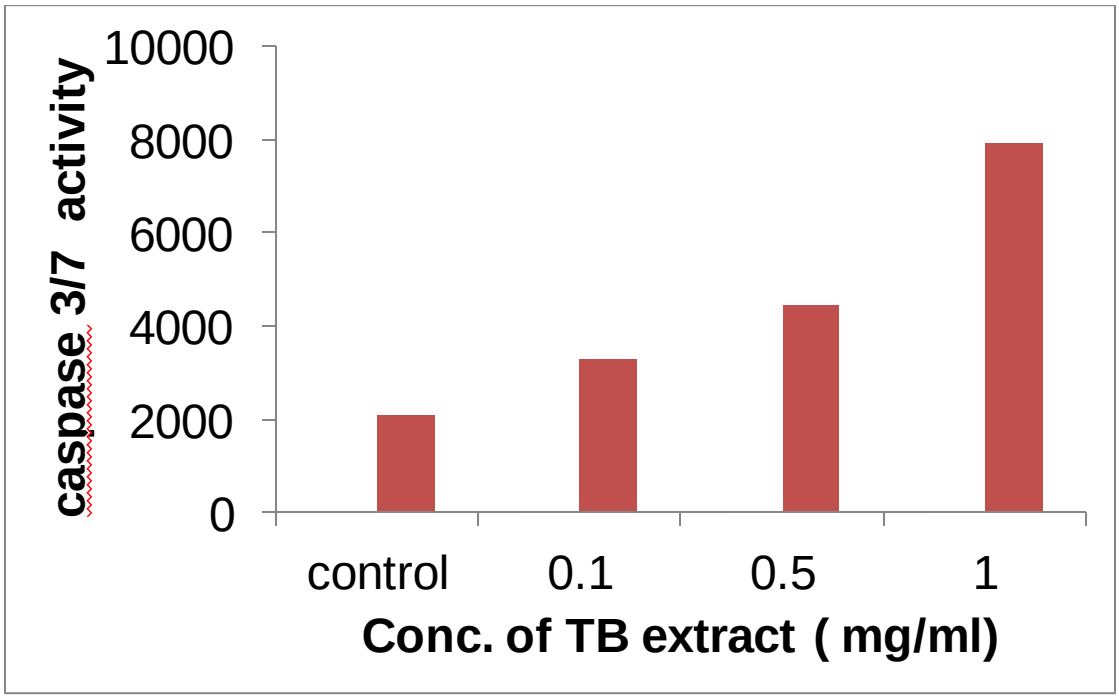


Fig- 5: Graph showing caspase 3/7 activity of TBE treated Hep 2 cell

Inference:

It can be inferred from this graph that, with increase in the concentration of TBE, the cells show increased caspase 3/7 activity which ultimately proves that apoptotic activity is increasing in a dose dependent manner.

6.5. Acridine orange staining:

Acridine orange staining was performed to see whether the presence of autophagy or not in the TBE treated Hep 2 cells.

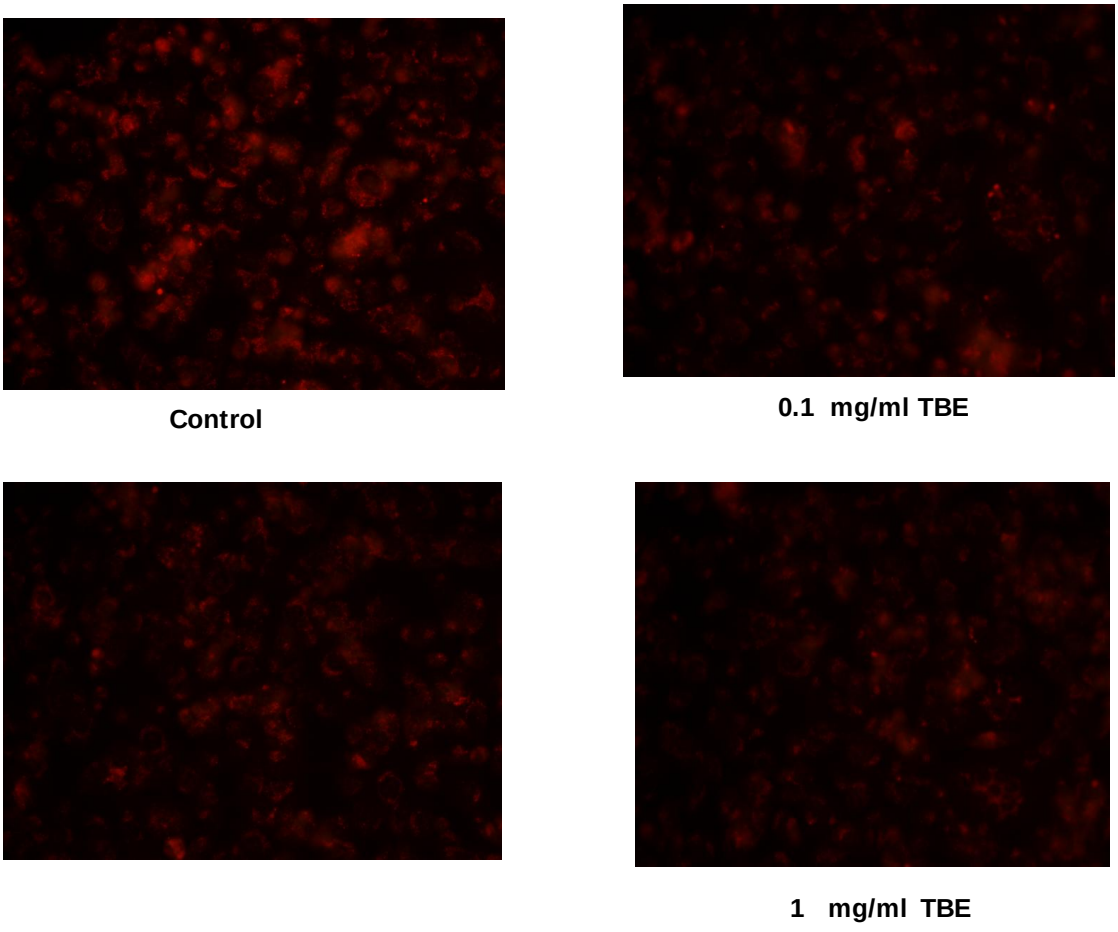


Fig-6: Acridine orange staining of control and TBE treated Hep 2 cells

Inference: From the above figures, it can be seen that intensity of red colour is decreasing which indicates decrease in autophagic vacuole or late autophagosomes in TBE treated cells with increase in the concentration of TBE dose whereas the microscopic observationof the cells showed the increase in nonviable cells with increase in TBE dose treatment. So, interestingly, it can be inferred that there was previousely protective autophagy machinery employed by the Hep 2 cells for their survival, proliferation and metastasis, but TBE inhibits protective autophagy in the Hep 2 cells. **6.6. Comet assay :**

Comet assay was performed to detect dsDNA damagein Hep 2 cells when treated with TBE.

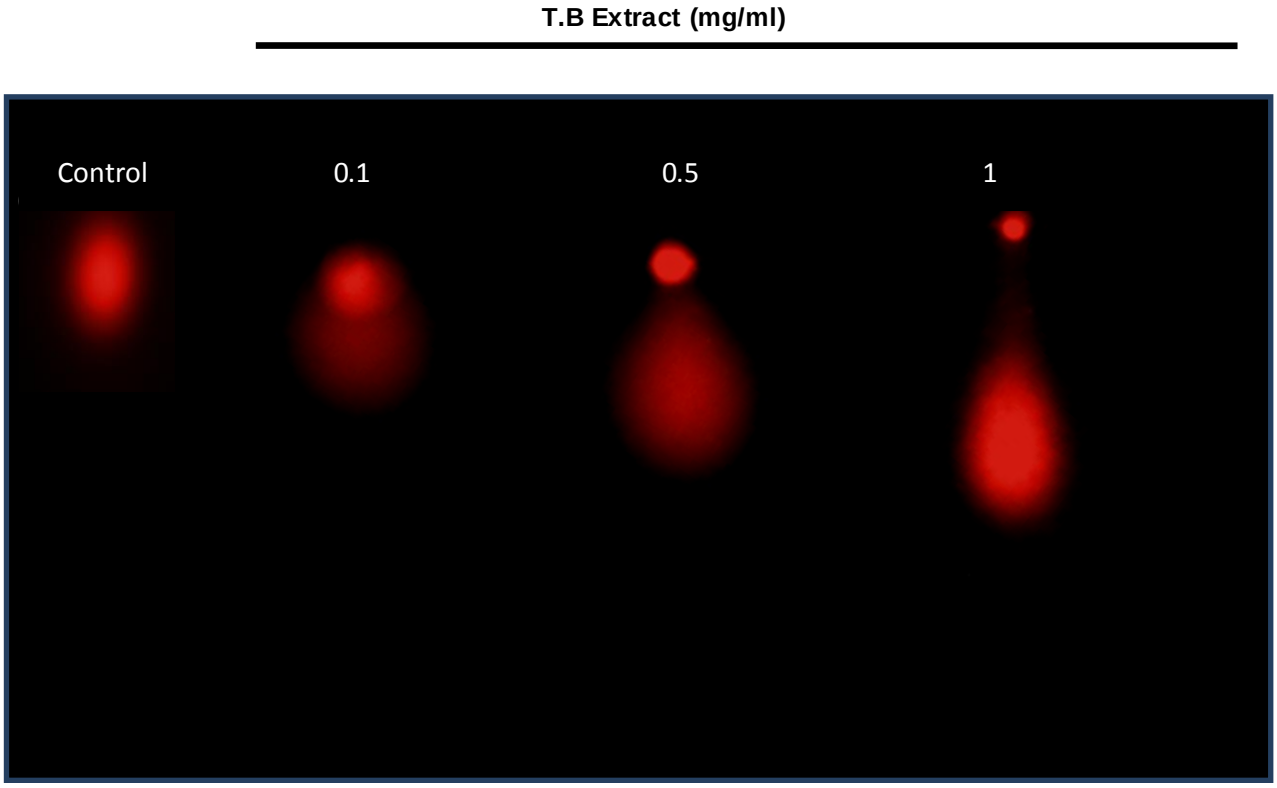


Fig- 7: The head and tail moment of a comet due to the effect of TBE

Inference :

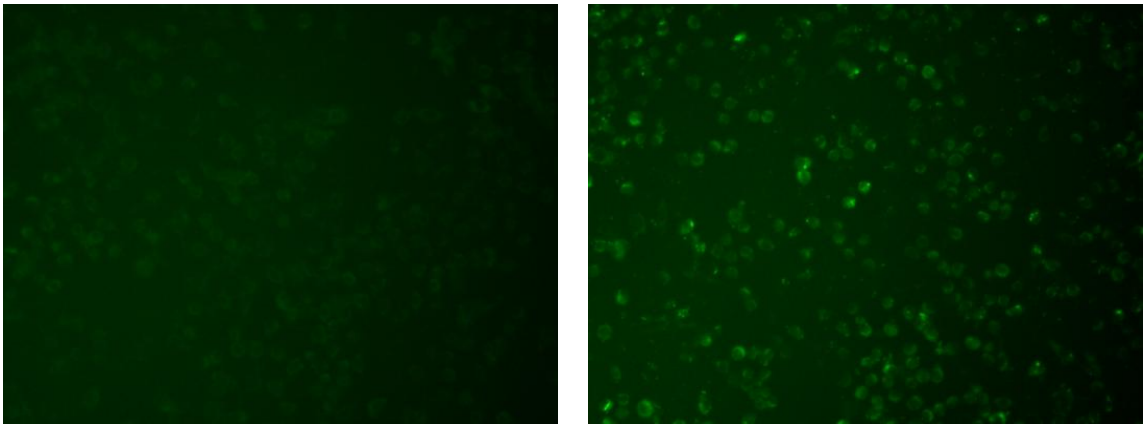
The increase in tail length and more accumulation or more intensity at the tail region conforms the DNA damage in Hep 2 cells with increase in the dose of TBE.

6.7. Determination of reactive oxygen species (ROS) by dihydrorhodamine:

To detect the ROS activity of different concentration of TBE treated Hep 2 cells and to detect ROS mediated Apoptosis in the cells, the cells treated with different concentrations of TBE were treated with dihydrorhodamine 123 and the results were seen under fluorescent microscope.

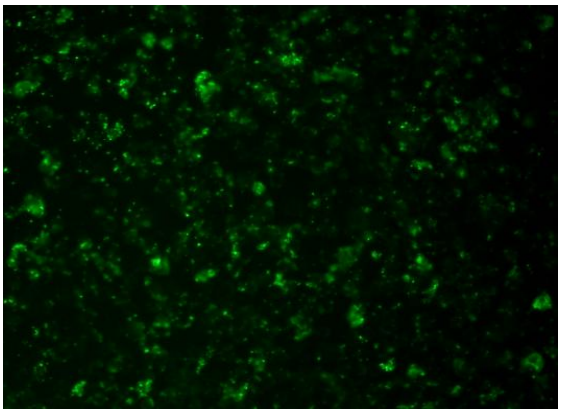
Inference:

The increasing trend of green intensity from control to TBE treated cells shows that after TBE treatment, the cells possibly undergo ROS mediated apoptosis. Production of ROS increases with increase in the dose of TBE (Fig -8).

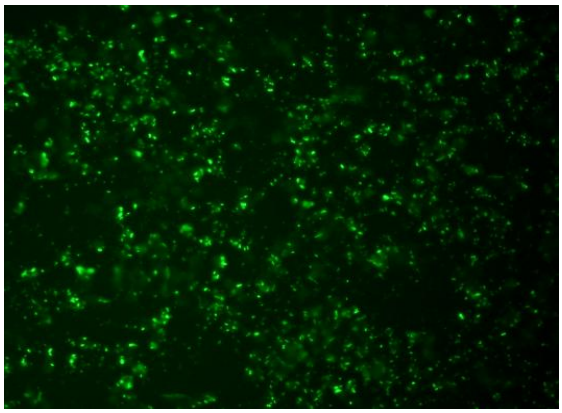


control

0.1 mg/ml TBE



0.5 mg/ml TBE



1 mg/ml TBE

TBE- *T. bellerica* extract

Fig-8 : Treatment of Hep 2 cells with dihydrorhodamine to detect ROS mediated apoptosis

So, in summary it can be predicted that, an active molecule in the TBE plays a significant role in inhibiting protective autophagy (from Acridine orange staining staining results) and induces apoptosis (from ROS test, DAPI staining, Caspase 3/7 glo assay, Comet assay, DNA laddering test) in Hep 2 cells, thus inhibiting the unabated proliferation of the cancer cells (Hep 2).

7. CONCLUSION

It has remained fascinating for scientists to eliminate tumors by manipulating autophagy [47]. Inhibiting or promoting autophagy therapeutically for cancer treatment will be a great approach to anticancer drug development. The molecular interplay between autophagy and cancer is also a less studied discipline for cancer therapy. The mechanism of regulation or deregulation of autophagy in cancer is different in different types of cancer cases. For example, in the anti-angiogenesis therapy, the main reason behind its failure is that, cancer cells trigger autophagy in response to hypoxia after this therapy and tend to survive [48]. In this case if autophagy is inhibitited along with the administration of anti-angiogenic drug, then recurrence of cancer can be checked. In another case, activation of autophagy can be effective in cancer treatment, such as, the TNBC (triple negative breast cancer) derived cell lines are reported to have decreased cell viability when these are treated with mTOR pathway inhibitor, so that, inhibition of autophagy gets released [49]. Another thing is to deeply understand the relationship between the two programmed cell death mechanisms: autophagy and apoptosis in cancer development. In some cases, one of them is inhibited, another is induced, or both are induced, or both are suppressed, or one is independent of the other and vice versa. No doubt, extensive research in this regard can make way for highly effective cancer therapy, however, we are at the initial stage of such a huge success in the present scenario.

8. FUTURE ASPECTS

The future aspects of this study can bring a potential anti-cancer drug from a very cheap plant product which is already very popular among Indians. So many things are to be investigated for

an anticancer drug from *T. bellerica*. First, the active compounds in TBE are to be isolated and characterized which is responsible for anticancer property in oral cancer cells. Then, its role in crosslinking apoptosis and autophagy in cancer suppression is to be found out. And also the effective drug delivery pathway for *T. bellerica* is to be validated. If really, it will be proved that the fruit *Terminalia bellerica* has highly significant role in oral cancer treatment, then it will be a nice discovery in medical science for development of a cheap but effective anti-cancer medicine.

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